Impact of anthocyanin from *Malva sylvestris* on plasma lipids and free radical

WANG Zhen-yu [1,2]

[1] *Forestry College, Northeast Forestry University, Harbin 150040, P. R. China*

[2] *Food Science Academy, Harbin Institute of Technology, Harbin 150086, P. R. China*

Abstract: The effects of anthocyanin from *Malva sylvestris* on plasma lipids and free radical were investigated by Reagent Kit method and in vitro assay. High fat model was set up with albino rats that were fed with different dosages of anthocyanin from *Malva sylvestris* (0.03 g·d^{-1}, 0.04 g·d^{-1} and 0.05 g·d^{-1}). The results showed that the total cholesterol was decreased by 19.7% at an anthocyanin of 0.04 g·d^{-1} and triglyceride was decreased by 34.4% at an anthocyanin of 0.05 g·d^{-1}. In vitro assay, some indexes of anthocyanin were measured including the capability of scavenging free radical, reducing force and the capability of anti-lipid peroxidation by orthophenanthroline Fe^{+2} oxidation-reduction method. The results indicated that the clearance rate of free radical reached to 43.46% when the content of anthocyanin was 0.20 mg·mL^{-1} and the inhibition ratio of lipid peroxidation reached 18.82% when the content was 0.5 mg·mL^{-1}. Therefore anthocyanin is a kind of natural and effective free radical remover and antioxidant and can prevent from the formation of the thrombus and reduced the emergence of the cardiovascular disease.

Keywords: Anthocyanin; Plasma lipids; Free radical

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Introduction

Malva sylvestris, belonging to *Malva*, contains large quantity of anthocyanin, which is a kind of very important natural and functional pigment and has certain physiological functions to the human bodies, so it can be applied extensively in such fields as food, pharmacy and cosmetics. With the development of medical technology, people come to know that the key factor that causes arteriosclerosis and cardiopathy is the rise of both low-density lipoprotein—C and cholesterol in the body (Ghiselli *et al.* 1998). The anthocyanin showed very effective treatment on hypertension caused by high plasma lipids level (Meunier *et al.* 1987). Kayamori *et al.* (1994) fed rats with food containing a large amount of cholesterol, simultaneously feeding them with an acyl-delphinidin extracted from eggplant, and then found that the total cholesterol in serum dropped, while the cholesterol and bile acid in the excrement increased. It indicated that the anthocyanin partly hindered small intestines from absorbing cholesterol and bile acid (Kayamori *et al.* 1994). Additionally the anthocyanin extracted from the grape can also reduce the levels of the cholesterol and low-density lipoprotein—C effectively (Ross 1993; Zhang *et al.* 2002). Besides function of reducing plasma lipids, anthocyanin also has the strong anti-oxidizing property that was found in the 1990s. Some researches on anthocyanin extracted from the wild grape showed its extremely strong anti-oxidizing property (Igarashi *et al.* 1994). The experiments about the anti-oxidant activity of noctilux-3-glucoside and pigment from cornflower showed that they also had extremely strong anti-oxidizing property (Tsuda *et al.* 1994). Postgraduate Research Institute of French Academy has ever studied on the ac-

tive capability of anthocyanin extracted from grape kernels to scavenge free radicals and the result showed that this anthocyanin was the most effective free-radical scavenger up to now. Sugimoto in Japan has verified that anthocyanin has the function of scavenging free radicals and the clearance rate can be up to 94%. Ling Zhiqun (2002) found that the right amount of anthocyanin had the function of resisting the active oxygen (Fang *et al.* 2001). The high-purity anthocyanin from grapes has the function of scavenging free radicals and it could scavenge 80% hydroxyl radicals with the concentration of 10 g·L^{-1}. So it is of great benefit to find the proper extrinsic antioxidant to scavenge the free radicals from the body, treat the disease, and keep the people health (Yu *et al.* 2002). The chemical compounds with the flavonoids structure have been a hotspot to the people in terms of the study on scavenging the free radical (Li *et al.* 2000). Anthocyanin has the typical flavonoids structure. In this study, the impacts of anthocyanin from *Malva sylvestris* on plasma lipids were investigated. And study on scavenging hydroxyl free radicals and lipid free radicals had positive meanings to preventing free radicals' oxidization, protecting people from the disease and keeping them health.

Materials and methods

Materials

Anthocyanin sample was homemade from *Malva sylvestris*. The reagents were provided by Harbin JiaShi Biotech. Co. Ltd., including 4-chlorphenol, potassium ferrocyanide, Adenosine Triphosphate (ATP), lipoprotein lipase (LPL), phosphor-glycerol oxidase (GPO), the glycerokinase (GK), peroxidase (POD), cholesterol esterase (CHE), cholesterol oxidase (CHOD), 4-AAP (amino-antipyrine), lipidosome-PBS (phosphate buffer solution with KH$_2$PO$_4$ and NaOH) decentralized system (LLS), Lecithin, 3-trichloroacetic acid (TCA), thiobarbituric acid (TBA), glacial acetic acid, orthophenanthroline and 3 standard-samples of triglyceride, total cholesterol and high-density lipoprotein—C. Reagent R$_1$, R$_2$, R$_3$, R$_4$, R$_5$ and R$_6$ were formulated as follows.

Reagent R_1 was prepared with 0.15-mmol·L^{-1} Tris (tris-hydroxymethyl aminomethane) buffer (pH 7.6), 3.5-mmol·L^{-1} 4-chlorphenol and 6-μmol·L^{-1} potassium ferrocyanide.

Reagent R_2 was prepared with more than 0.15-mmol·L^{-1} ATP, 0.35-mmol·L^{-1} 4-AAP, 3000-u·L^{-1} LPL, 2500-u·L^{-1} GPO, 200u·L^{-1} GK, 150-u·L^{-1} POD and 2.26-mmol·L^{-1} standard-sample of triglyceride.

Reagent R_3 was prepared with 1140-u·L^{-1} CEH, 800-u·L^{-1} CHOD and 6000-u·L^{-1} POD.

Reagent R_4 was prepared with 100-mmol·L^{-1} phosphate buffer (pH 7.7), 3.5-mmol·L^{-1} 4-chlorphenol, 0.5-mmol·L^{-1} 4-AAP and 2.59-mmol·L^{-1} standard-sample of cholesterol.

Reagent R_5 was prepared with 100-mmol·L^{-1} GOOD'S buffer (pH 7.0), 5-mmol·L^{-1} HSDA and 1.5-mmol·L^{-1} Mg^{2+}.

Reagent R_6 was prepared with 100-mmol·L^{-1} GOOD'S buffer (pH 7.0), 5-ku·L^{-1} CEH, 5 -ku·L^{-1} CHOD, 3-ku·L^{-1} POD, 0.25-mmol·L^{-1} 4-AAP and 2.50-mmol·L^{-1} standard-sample of HDL—C.

Methods

Fifty 120-day-old albino rats, with weight of 300 g each, were divided into five groups (10 rats in each group), such as control group, hyper-lipid fodder group, and three dosage-groups. Control group was fed with basic fodder, hyper-lipid fodder group with only hyper-lipid fodder, and three dosage-groups were fed with hyper-lipid fodder and anthocyanin.

Hyper-lipid fodder formulation consisted of 93.8% basic fodder, 1% cholesterol, 5% lard oil and 0.2% cholate.

Fodder sample containing 0.03-g anthocyanin was designed to feed each 300-g-weight rat in dosage-group 1 each day, which was about 6 times more than daily intake dose of anthocyanin (0.0167 g·kg^{-1}) per capita, (Yang 1997).

Fodder sample containing 0.0401-g anthocyanin was designed to feed each 300-g-weight rat in dosage-group 2 each day, which was 8 times more than the above reference figure (0.0167 g·kg^{-1}).

Fodder sample containing 0.0501-g anthocyanin was designed to feed each 300-g-weight rat in dosage-group 3 each day, which was 10 times more than the above reference figure (0.0167 g·kg^{-1}).

All rats at 25(±2) °C in a draughty and sunny condition were fed with fodder twice every day for 14 days. At the 15th day, caudal blood of 1 mL was carried on biochemical determination by Reagent Kit method with the use of 752 ultraviolet spectrophotometer and 721 spectrophotometer, including the determination of total cholesterol (TC), triglyceride (TG) and high-density lipoprotein—C (HDL—C).

Determination of the effect of anthocyanin on TG (triglyceride)

Caudal blood from albino rats was centrifuged and 0.01-mL serum was put in test tube with 0.55-mL reagent R_1, and 0.45-mL R_2. The intermixture was mixed completely at 37 °C for 10 min. The absorbance (As) was measured at 500 nm. The blank sample was composed of reagent R_1, R_2 and 0.01-mL distilled water. The absorbance of standard solution of TG (A_0) was measured by the same above procedure. The concentration of TG in serum (C) was obtained by Equation (1).

$$C = A_s \times \frac{2.26 \times 10^{-3}}{A_0} \tag{1}$$

where, C is the concentration of TG in serum from albino rat, (mol·L^{-1}); A_s is the absorbance of intermixture containing reagents and serum from albino rat fed anthocyanin; A_0 is the absorbance of intermixture containing reagents and standard-sample of TG; 2.26×10^{-3} is the concentration of standard-sample of TG.

Determination of the effect of anthocyanin on TC (total cholesterol)

The 0.01-mL serum was put in test tube and then 0.64-mL reagent R_3, 0.36-mL R_4 were added to it. The intermixture was vibrated and mixed completely at 37°C for 6 min. The absorbance (As) was measured at 500 nm by using the spectrophotometer (type 721). In the blank sample, 0.01-mL distilled water was in place of serum. The absorbance of standard solution of TC (A_0) was measured according to the same above procedure. The concentration of TC in serum (C) was obtained by Equation (2):

$$C = A_s \times \frac{2.59 \times 10^{-3}}{A_0} \quad (\text{mol·L}^{-1}) \tag{2}$$

where, C is the concentration of TC in serum from albino rat; A_s is the absorbance of intermixture containing reagents and serum from albino rat fed anthocyanin; A_0 is the absorbance of intermixture containing reagents and standard-sample of TC; 2.59×10^{-3} is the concentration of standard-sample of TC.

The determination of the effect of anthocyanin on HDL—C (high-density lipoprotein—C)

Caudal blood from albino rats (having fasting for 12h) was centrifuged for getting serum. The 4-μL serum was put in test tube and then 300-μL reagent R_5 was added to it. The intermixture was vibrated and mixed completely at 37°C for 5 min. The absorbance (A_1) was measured at 546 nm by the biochemical analyzer. Then 100-μL reagent R_6 was added, mixed completely and acted at 37°C for another 5min. The absorbance (A_2) was measured at 546nm. In the blank sample, 4-μL-distilled water was in place of serum. The absorbances of standard solution of HDL—C (A_{s1} and A_{s2}) were measured by the same above procedure. The concentration of HDL—C in serum (C) was obtained by Equation (3):

$$C = \Delta A \times \frac{2.50 \times 10^{-3}}{\Delta A_s} \quad (\text{mol·L}^{-1}) \tag{3}$$

$$\Delta A = A_2\text{-}A_1; \quad \Delta A_s = A_{s2}\text{-}A_{s1}$$

here, C is the concentration of HDL—C in serum from albino rat; ΔA_s is the difference of two absorbencies of intermixtures, containing reagents and serum from albino rat fed anthocyanin, before and after adding reagent R_6; ΔA_0 is the difference of two absorbencies of intermixture, containing reagents and standard-sample of TG, before and after adding reagent R_6; 2.50×10^{-3} is the concentration of standard-sample of HDL—C.

Determination of capability of anthocyanin's scavenging hydroxyl radicals (Zeng 1999)

The 1-mL orthophenanthroline (5mmol·L^{-1}) was aspirated

into test tube and 2-mL phosphate buffer $(0.75\text{mol} \cdot \text{L}^{-1})$ (pH 7.4) and 2-mL $FeSO_4$ solution $(7.5\text{mmol} \cdot \text{L}^{-1})$ were added with complete mixing, respectively. Then 1-mL double distilled water was used to supplement volume. 1-mL H_2O_2 (1%) was added (called injured test) or non-added (called uninjured test) and then the two tubes with H_2O_2 and without H_2O_2 were reacted at 37 °C for 60 min. The absorbencies at 536 nm were measured as A_1 and A_0. Additionally, 4 gradient concentrations of anthocyanin solution $(0.05 \text{ mg} \cdot \text{mL}^{-1}, 0.1 \text{ mg} \cdot \text{mL}^{-1}, 0.15 \text{ mg} \cdot \text{mL}^{-1}, \text{and } 0.2 \text{ mg} \cdot \text{mL}^{-1},$ respectively) were designed to be put in four tubes mentioned above (2-mL phosphate buffer $(0.75 \text{ mol} \cdot \text{L}^{-1})$, 2-mL $FeSO_4$ solution $(7.5 \text{ mmol} \cdot \text{L}^{-1})$, 1-mL double distilled water and 1-mL H_2O_2 of 1%). Then four test tubes were reacted at 37 °C for 60 min and measured the absorbance at 536 nm as A_{s1}, A_{s2}, A_{s3} and A_{s4}. The clearance rate of hydroxyl radicals (marked as CR) was calculated by the Equation (4).

$$C_R = \frac{A_s - A_1}{A_0 - A_1} \times 100\% \qquad (4)$$

where, C_R is the clearance rate of hydroxyl radicals; A_s is the absorbance of intermixture containing reagents and anthocyanin in different concentrations; A_1 and A_0 are the absorbencies of the intermixture with H_2O_2 and without H_2O_2, respectively.

Determination of capability of anthocyanin's inhibiting lipid peroxidation

Lecithin solution of 1 mL and anthocyanin sample of 1 mL were put into the test tube sequentially and mixed completely at 37 °C for 60 min. Then 2-mL TCA-TBA-HCl intermixture was added and reacted in water bath at 90–100 °C for 15 min. Then the tube was cooled rapidly and centrifuged at 2000 r · min⁻¹ for 10 min. The supernatant fluid was measured at the absorbance of 535 nm (As). The absorbances for 4 gradient concentrations of anthocyanin sample $(0.2 \text{ mg} \cdot \text{mL}^{-1}, 0.3 \text{ mg} \cdot \text{mL}^{-1}, 0.4 \text{ mg} \cdot \text{mL}^{-1},$ and $0.5 \text{ mg} \cdot \text{mL}^{-1},$ respectively) were marked as A_{s1}, A_{s2}, A_{s3}, A_{s4} (Mazza 1987). The absorbance of intermixture, containing 1-mL double-distilled water instead of 1-mL anthocyanin sample, was measured by the same above procedure (Ac). The capability of anthocyanin's inhibiting lipid peroxidation (marked as IR) was determined by the Equation (5).

$$I_R = \frac{A_c - A_s}{A_c} \times 100\% \qquad (5)$$

where, I_R is the capability of anthocyanin's inhibiting lipid peroxidation; A_s is the absorbance of intermixture containing reagents and anthocyanin in different concentrations; A_c is the absorbance of intermixture with 1-mL double-distilled water instead of 1-mL anthocyanin sample.

Results

Effect of anthocyanin from *Malva sylvestris* on triglyceride (TG)

The triglyceride contents of subjects in five groups were different (from 0.916 to 1.396 mmol · L⁻¹) with feeding various content of anthocyanin. With increasing the content of anthocyanin, the content of triglyceride was decreased (Fig. 1). Furthermore, by the test of significance difference (Table 1), effect of anthocyanin

from *Malva sylvestris* on triglyceride in dosage-group 3 was great significant $(t=30.36>t_{0.01\ (9)}=3.250)$.
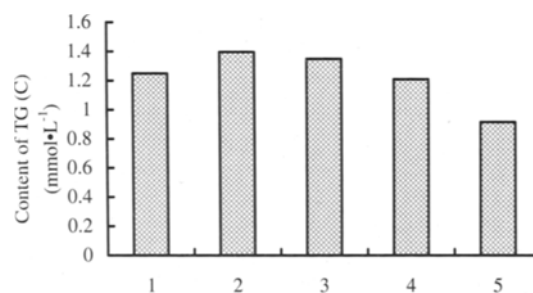

Fig. 1 Effect of anthocyanin on triglyceride in different dosages
1—control group, 2—hyper-lipid fodder group, 3—dosage-group 1 fed with 0.03g of anthocyanin, 4—dosage-group 2 fed with 0.04g of anthocyanin, 5—dosage-group 3 fed with 0.05g of anthocyanin.

Table 1. Effect of anthocyanin from *Malva sylvestris* on triglyceride in difference groups

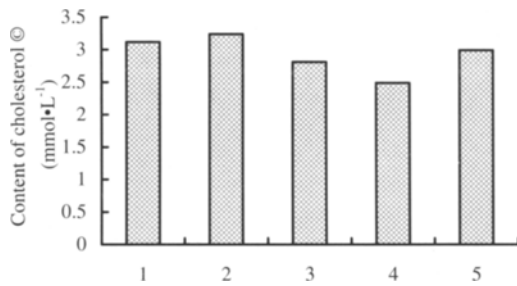| Group	Difference $(\bar{d})$	Standard deviation (s)	Freedom (df)	t	$t_{0.01}$ (%)
Dosage-group 1	0.0978	0.026	9	3.761	
Dosage-group 2	0.0316	0.0139	9	2.27	3.250
Dosage-group 3	0.334	0.011	9	30.36**	

Effect of anthocyanin from *Malva sylvestris* on cholesterol

All the 3 dosage-groups had the function of reducing cholesterol, compared with the control group $(3.108 \text{ mmol} \cdot \text{L}^{-1})$ and hyper-lipid fodder group $(3.214 \text{ mmol} \cdot \text{L}^{-1})$ (Fig. 2), of which, the effect in dosage-group 2 $(2.496 \text{ mmol} \cdot \text{L}^{-1})$ was the relatively strongest and in dosage-group 3 $(2.996 \text{ mmol} \cdot \text{L}^{-1})$ was the weakest. It is proved that the more anthocyanin was fed, the more obviously cholesterol was reduced. However, with the quantity further increasing, the reverse effects appeared. In dosage-group 3 the average content of cholesterol had an upward tendency, which was slightly lower than that in the hyper-lipid fodder group.

The cholesterol content had a downward trend, which of dosage-group 1 and dosage-group 2 was 9.7% and 19.7%, respectively. It is indicated that the content of anthocyanin fed in dosage-group 2 can reduce the content of total cholesterol in serum. However, the rate of descent of the total cholesterol in dosage-group 3 was 3.6%. Furthermore, by the test of significant difference (Table 2), effects of anthocyanin from *Malva sylvestris* on cholesterol in all the 3 dosage-groups were great significant.

### Effect of anthocyanin from *Malva sylvestris* on high-density lipoprotein—C (HDL—C)

The average contents of high-density lipoprotein—C (HDL—C) in test groups have a downward trend, compared with that in control group $(1.228 \text{ mmol} \cdot \text{L}^{-1})$, (Fig. 3), of which, HDL—C of dosage-group 2 $(0.654 \text{ mmol} \cdot \text{L}^{-1})$ reduced most significantly. By the test of significant difference (Table 3), effects of anthocyanin from *Malva sylvestris* on high-density lipoprotein—C in dosage-group 1 and dosage-group 2 were more significant $(t_{0.01\ (9)}=3.250)$, which were the most significant in dosage-group 3.

Fig. 2   Effect of anthocyanin on cholesterol in different dosages

1—control group, 2—hyper-lipid fodder group, 3—dosage-group 1 fed with 0.03g of anthocyanin, 4—dosage-group 2 fed with 0.04g of anthocyanin, 5—dosage-group 3 fed with 0.05g of anthocyanin.

Table 2.   Effect of anthocyanin from *Malva sylvestris* on cholesterol in difference groups

Group	Difference ($\bar{d}$)	Standard devia-tion(s)	Freedom (df)	t	$t_{0.01}$ (%)
dosage-group 1	0.41	0.019	9	21.57**	
dosage-group 2	2.006	0.021	9	95.52**	3.250
dosage-group 3	2.298	0.027	9	85.11**	
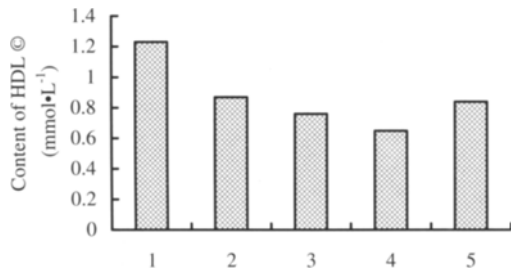
Fig. 3   Effect of anthocyanin on high-density lipoprotein—C in different dosages

1—control group, 2—hyper-lipid fodder group, 3—dosage-group 1 fed with 0.03g of anthocyanin, 4—dosage-group 2 fed with 0.04g of anthocyanin, 5—dosage-group 3 fed with 0.05g of anthocyanin.
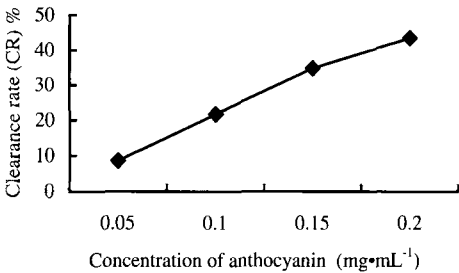
Table 3. Effect of anthocyanin from *Malva sylvestris* on high-density lipoprotein—C in difference groups

Group	Difference	Standard deviation(s)	Freedom (df)	t	$t_{0.01}$ (%)
dosage-group 1	0.022	0.00578	9	21.10**	
dosage-group 2	0.224	0.0038	9	58.94**	3.250
dosage-group 3	0.029	0.0036	9	8.056*	

Effect of anthocyanin from *Malva sylvestris* on scavenging hydroxyl radicals

From the experimental result, six absorbance values were obtained (A_{s1} 0.231, A_{s2} 0.234, A_{s3} 0.237, A_{s4} 0.239, A_1 0.229 and A_0 0.252). By calculating, the rate of scavenging free radicals at an anthocyanin concentration of 0.05 mg·mL^{-1} was the lowest (8.6%) and was the highest (43.46%) at an anthocyanin concentration of 0.20 mg·mL^{-1} (Fig. 4). It was also found that the con-

centration of anthocyanin presented direct relation with the rate of scavenging free radicals. It was indicated that anthocyanin from *Malva sylvestris* had the capability of scavenging hydroxyl radicals.

Fig. 4   Curve of anthocyanin's scavenging hydroxyl radicals in different concentration

Effect of anthocyanin from *Malva sylvestris* on inhibiting lipid peroxidation

Five absorbance values were obtained, such as Ac 1.440, As_1 1.330, As_2 1.260, As_3 1.246 and As_4 1.169, at different concentrations of anthocyanin. Then the inhibition rates were calculated by Equation (5). Of the results, the inhibition rate was the lowest (9.72%) when the anthocyanin concentration was 0.2 mg·mL^{-1} and was the highest (18.82%) when the anthocyanin concentration was 0.5 mg·mL^{-1} (Fig. 5). It was also found that the inhibition rate would rise with increasing the concentration of anthocyanin.
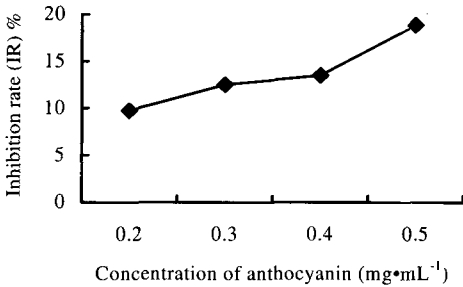
Fig. 5   Curve of anthocyanin's inhibiting lipidosome in different concentration

Discussion

The free radical refers to the atom, atomic group, molecule and ion with the unpaired valence electron. The excessive free radicals will do harm to such bio-macromolecules in the human bodies as lipid, nucleic acid, saccharide, protein, etc. and lead to a lot of diseases, for instance, apoplexy, pulmonary emphysema, cataract even cancer, etc. The anthocyanin is one of the Flavonoids, which can inhibit the peroxidation of the lipid, scavenge free radicals and prevent hyperlipemia and cardio-cerebral angiopathy caused by them (Zheng 2000).

According to the result on anthocyanin regulating the plasma lipids, a right intake of anthocyanin from *Malva sylvestris* can reduce the total cholesterol and triglyceride in the blood of the subjects, so that it leads to prevent the formation of the thrombus and to reduce the emergence of the cardio-cerebral angiopathy, which was consistent with the results of studies made by Ka-

yamori *et al.* (1994), Ross (1993) and Zhang *et al.* (2002). And there are four types of lipoprotein in body, of which HDL—C can reduce the risk of atherosclerosis and coronary disease largely. So the level of HDL—C in body can be improved to decrease the coronary disease (Zhang *et al.* 2004). This study did not get an ideal result about the effect of anthocyanin to all dosage on HDL—C. So a further study on dosage and HDL—C content is suggested to do in order to decide the function of anthocyanin from *Malva sylvestris* in preventing atherosclerosis and coronary disease.

The non-oxidizability of anthocyanin sample with the catalysis of Fe ion may be a synthetic representation of both chelating Fe ion and scavenging free radicals. Because of the capability of chelating Fe ion, the chelation ratio will be upward with the dosage of the sample increasing in the reaction system, which makes the initial step of lipid peroxidation slow down and free radicals produced from the reaction with hydroperoxides reduce relatively. It is indicated in this study that anthocyanin from *Malva sylvestris* is one natural and effective free-radical scavenger and antioxidant, which was consistent with the results from Igarashi *et al.* (1994) and Tsuda *et al.* (1994).

Conclusions

The anthocyanin from *Malva sylvestris* had better properties of regulating plasma lipids. By the animal experiments, 0.03 g·d^{-1} anthocyanin fed to each albino rat could reduce the content of triglycerides and 0.0401-g·d^{-1} anthocyanin could reduce the total cholesterol in serum.

The anthocyanin from *Malva sylvestris* had extremely strong capability of scavenging free radicals. The clearance rate of free radical reached to 43.46% when the content of anthocyanin was 0.20 mg·mL^{-1} and the inhibition ratio of lipid peroxidation was 18.82% when the content reached 0.5 mg·mL^{-1}.

Therefore, the anthocyanin from *Malva sylvestris* was one natural and effective free-radical scavenger and antioxidant. It can be applied widely to both preventing and treating the diseases caused by free radicals.

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